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Review

Viral reservoirs in elite controllers of HIV-1 infection: Implications for HIV cure strategies

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ABSTRACT

Elite controllers are HIV-1 positive subjects who control viral replication without antiretroviral therapy. Many of these subjects have replication-competent virus and thus represent a model of a functional cure. Peripheral CD4+ T cells in these subjects have small reservoirs with a low frequency of intact proviruses. Furthermore, recent studies suggest that many of these intact proviruses are disproportionally integrated at sites that have limited transcriptional activity raising the possibility that replication-competent viruses do not replicate because they are in a "blocked and locked" state. However, this feature is probably a consequence rather than a cause of elite control. Additionally, evolution of plasma virus has been detected in many elites suggesting that there continues to be ongoing viral replication in other compartments. While exceptional elite controllers with very limited viral reservoirs have recently been described, more work is needed to determine whether these patients have achieved a sterilizing cure.

1. Introduction

Latently infected CD4+ T cells are quiescent memory CD4+ T cells that have the viral genome integrated into the host genome. While viral replication does not occur in these resting CD4+ T cells, activation can lead to viral transcription and new rounds of viral replication. Thus, these cells represent the major barrier to HIV eradication [1]. There are two possible ways of curing HIV; a sterilizing cure involves the eradication of all replication-competent HIV proviruses whereas a functional cure involves controlling HIV replication without antiretroviral therapy. HIV controllers are subjects who are models of a functional cure [2]; many are infected with replication-competent virus, but they control viral replication without antiretroviral therapy. A large percentage of these patients have protective HLA alleles [3,4] and potent T cell responses [5] that are thought to play a role in controlling viral replication. However, HIV controllers are a heterogeneous group of patients and some do not have protective HLA alleles or HIV-specific immune responses. Multiple factors that have not yet been fully characterized, including viral fitness, may contribute to elite control in these subjects. HIV controllers are divided into 2 categories based on the degree of control: elite controllers (ECs) maintain viral loads below the limit of detection of commercial viral load assays and viremic controllers (VCs) have viral loads that are detectable but less than 2000 copies/ml [6]. A better understanding of the viral reservoir in these subjects is needed if a functional cure is to be achieved in patients with progressive disease who are on suppressive antiretroviral (ART) regimens (chronic progressors, CPs). New technology has led to improved characterization of the viral reservoir in CPs, and several recent studies have applied these methods to ECs. Here we review recent advances in our understanding of the viral reservoir in ECs and discuss the lessons we can apply to CPs.

2. Evidence of replication-competent virus in ECs

The Sydney Blood Bank cohort is made up of individuals who were infected by blood transfusions from a patient who was infected with an HIV-1 isolate that contained large deletions in the nef gene and the LTR. All of the recipients became slow progressors with low levels of viremia or ECs [7]. More recently, a patient who was infected with a vpr-defective HIV-1 molecular clone and became an EC has been described [8] and members of a transmission cluster of ECs have been found to be infected with HIV-1 isolates that contain related attenuated env genes [9]. Furthermore, the high percentage of people living with HIV-2 who become controllers suggests that viral factors may play a role in clinical outcomes [10]. Taken together, such studies suggest that infection with attenuated or defective viruses can lead to elite control, but they do not indicate that all ECs are infected with defective virus. Although it has been challenging to isolate replication-competent virus from most ECs, several labs have successfully isolated virus from a subset of these patients using some

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version of the quantitative viral outgrowth assay (QVOA, [11–15]). In one study, full genome sequence analyses of replication-competent isolates from 4 ECs showed no evidence of large deletions, hypermutations, or known attenuating mutations and phenotypic analyses also proved that these viruses were as fit as laboratory HIV-1 isolates [11]. Isolates from some EC have also been shown to replicate vigorously and cause CD4+ T cell depletion in humanized mice [16]. Furthermore, replication-competent virus has been isolated from both members of four HIV controller/CP transmission pairs [17–19] and comparable viral fitness from both members was seen in 3 out of 4 of these pairs [18,19]. Interestingly, in one of these studies, the pattern of escape mutations present in HLA-B*57 restricted Gag epitopes suggested that the elite controller had transmitted virus to the patient who became a CP [18]. Finally, there have been reports of ECs transiently [20] or permanently [19,21,22] losing control of viral replication and becoming viremic. Thus, it has become clear that some ECs are capable of controlling viruses that are fully replication-competent.

2.1. Characteristics of EC proviruses and integration sites

While many of the studies described above activated CD4+ T cells in QVOAs to isolate replication-competent virus, other studies have performed molecular analyses on unmanipulated CD4+ T cells. A low frequency of total [13] and integrated [23] HIV DNA has been reported in studies using standard PCR assays, however PCR does not distinguish between defective and infectious proviruses. A study based on more than 400 near full-length sequences of provirus from 28 CPs demonstrated that only 2.4% of proviruses in CPs did not contain large deletions or hypermutations and thus were said to be "intact" [24]. It is important to note that not all intact proviruses are replication-competent, as mutations and small insertions and deletions in critical areas of the viral genome can compromise viral fitness. However, some intact proviruses have been shown to replicate *in vitro* and thus intact proviruses represent a good surrogate for replication-competent virus [24]. Recently Bruner et al developed a high throughput intact proviral DNA assay (IPDA) that approximates the frequency of intact proviruses as determined by full genome sequencing [25]. Kwaa et al used the IPDA to compare the size of the viral reservoir in patients on ART and ECs and found that the frequency of both total and intact DNA in EC was approximately 20-fold lower than the frequency seen in patients on ART [26]. Jiang and colleagues sequenced 1,385 and 2,388 full length proviral genomes from 64 ECs and 41 CPs respectively and also found significantly lower levels of intact and total DNA in ECs [27]. Interestingly, the ratio of intact to total DNA was the same or higher in ECs compared to subjects on ART in the 2 cohorts of patients suggesting that ECs do not have a higher portion of defective virus.

Sequence analysis revealed no evidence of enrichment of escape mutations [28] or gross genetic defects [29] in proviral DNA from ECs compared to CPs. Digital PCR studies enabled the amplification and sequence analysis of clonal proviruses isolated from peripheral CD4+ T cells. Interestingly, proviral Gag [30] and Env proviruses [31] showed evidence of identical sequences which was unexpected given the low fidelity of HIV polymerase. It is now clear that this phenomenon is due to clonal expansion of infected CD4+ T cells where integrated viral genomes are copied by host DNA polymerase during cell proliferation [32]. Boritz et al analyzed 99 sequences from env in a cohort that was largely made up of viremic controllers and showed that the frequency of expanded clones ranged from 32.7% to 96.8% of sequences from peripheral CD4+ T cells [33]. Veenhuis et al found a comparable frequency of expanded env clones in ECs and showed that the frequency is higher in EC than in subjects on ART [34]. While one cannot determine whether clonally expanded virus is defective or replication-competent by sequencing individual genes, full genome sequence analysis of multiple viral isolates obtained from a culture assay from an EC with a large reservoir proved that there was clonal expansion of replication-competent virus. Jiang and colleagues also showed that there was clonal expansion of genome-intact viruses in EC and the frequency of these presumed replication-competent clonally expanded viruses was higher in EC than in subjects on ART [27]. It is possible that this enrichment of clonally expanded proviruses in ECs may be due to the fact that while viral replication results in the synthesis of viral proteins that can be identified by the immune system, clonal expansion can take place without antigen expression or virus production [35,36]. Thus, HIV-specific immune responses are more likely to eradicate CD4+ T cells containing actively replicating virus than infected CD4+ T cells that have undergone clonal expansion (Fig. 1A). Interestingly, Antar and colleagues did not find an enrichment of clonally expanded proviruses in 3 ECs on ART[37]. This could be due to the fact the initiation of ART in ECs leads to a reduction in the frequency of HIV-specific CD8+ effector T cells [14,38] which would lead to less selective pressure in these individuals.

Integration site analysis in HIV controllers was first performed by Boritz et al [33]. Approximately 90% of all env clones amplified from peripheral CD4+ T cells were integrated into NSD1 gene in one subject and in the POLM gene in another. Veenhuis et al showed that in an elite controller with a large reservoir, 15% of proviral clones amplified from 60 peripheral CD4+ T cells were integrated at the same site in WNK1, and 10% were integrated in the same site in ZNF470 [34]. Neither study distinguished between replication-competent and defective proviruses, but Jiang et al. used matched integration site and proviral sequencing [39] to analyze both integration sites and the corresponding proviral sequence [27]. They studied 92 integration sites from intact proviruses from 11 ECs and showed that 40% of proviral clones from ECs were integrated into non-genic or pseudogenic regions compared to 13% of intact proviral clones from subjects on ART. In addition, there was a significant enrichment of integration sites that occurred either in satellite DNA or in genes in the zinc-finger protein family, compared to integration sites in patients on ART. This is noteworthy as HIV integration rarely occurs in satellite DNA and this would be expected to lead to a higher level of transcriptional repression in ECs. Indeed, the authors found a lower ratio of viral transcripts to viral DNA in EC subjects compared to subjects on ART which confirms prior studies showing low levels of cell associated HIV RNA in ECs [40,41].

The authors show that superinfecting CD4+ T cells from elite controllers with a lab strain leads to a normal integration pattern [27] so it is unlikely that the majority of these individuals are infected with virus that happened to integrate at sites with heterochromatin features. It is much more likely that strong immune responses in ECs selectively eliminate CD4+ T cells that harbor transcriptionally competent virus over time due to expression of viral proteins in these cells. This is consistent with a study that found that latency reversal in a primary cell model was rare and depended on integration in an open chromatin context [42]. Selective immune mediated elimination of these transcriptionally-competent cells would lead to an enrichment of CD4+ T cells that harbor proviruses that are in a state of deep latency and thus do not express any viral proteins (Fig. 1B). This state of deep latency probably contributes to control of viral replication and may also provide an explanation for why it has been so challenging to culture virus from many ECs since immune activation with T cell mitogens may not lead to viral transcription and latency reversal in many infected cells.

3. Evidence of ongoing viral replication in ECs

The question then arises, if the majority of remaining proviruses in elite controllers exist in a 'locked and blocked state', is there any viral replication occurring in these subjects? The answer appears to be yes. Many laboratories have detected and quantified virus in the plasma of these subjects [43–46] and sequence analysis shows a

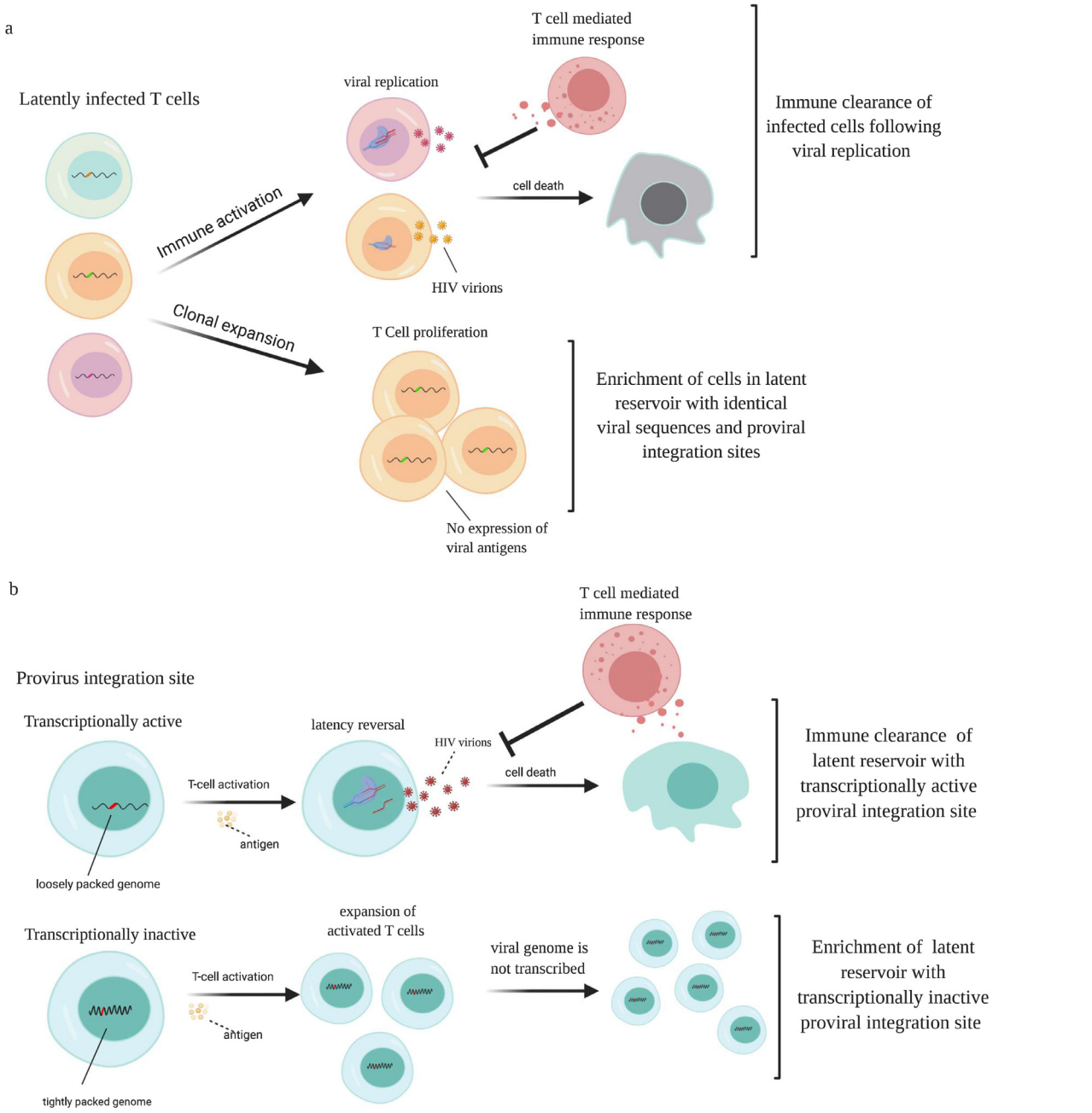

Fig. 1. Effect of clonal expansion and integration sites on the viral reservoir: A) Activation of latently infected T cells will lead to viral transcription and replication. The synthesis of viral protein will lead to immune recognition and elimination of these cells by the immune system. In contrast, clonal expansion of latently infected cells is unlikely to lead to viral transcription and replication, and will not trigger an immune response, leading to enrichment of cells with identical viral sequences and proviral integration sites. B) Proviruses can be integrated into transcriptionally active or inactive sites. Upon activation, cells with provirus integrated into transcriptionally active sites will make viral proteins and will be eliminated by the immune system. In contrast, cells with provirus integrated into transcriptionally inactive sites will not make viral proteins and can evade the immune system. This will lead to an accumulation of cells with proviruses that are integrated into transcriptionally inactive sites in ECs.

major discordance between plasma virus and virus in resting CD4+ T cells [30,47]. While virus from CD4+ T cells rarely have escape mutations, virtually every plasma virus amplified in Gag of HLA-B*57 ECs has at least one escape mutation. The presence of escape mutations in Gag in plasma virus has been confirmed [48] as has the discordance between proviral and plasma sequences in ECs [33]. This discordance suggests two things: 1) strong selective pressure is being exerted on viruses in these subjects and 2) virus replication is taking place in some compartment. Further evidence of ongoing viral replication in HLA-B*57 elite controllers comes from studies by O'Connell and colleagues. While no evolution was detected in provirus in peripheral CD4+ T cells in elite controllers, there was evolution of plasma virus in the same subjects over the same time frame [49,50]. Additionally, the proviral sequences were ancestral to the plasma sequences. This evolution of plasma virus was seen in a separate study in a different cohort of elite controllers where protective HLA alleles were not as overrepresented [51]. Studies in the monkey model of elite control suggest that the ongoing viral replication may be taking place in B cell follicles in lymphoid tissue [52] where CD8+ T cells are generally excluded [53]. Taken together these results suggest that the presence of deep latency seen in peripheral CD4+ T cells does not mean that viral replication is completely inhibited in these subjects. And while some studies have suggested that plasma virus from ECs are less fit than plasma virus from patients on ART

[48,54–57], the fact that viral evolution occurs proves that the virus is capable of ongoing replication [49–51].

4. Exceptional elite controllers

"Exceptional elite controllers" (EECs), are a heterogeneous group of patients who may have achieved a higher degree of viral control, and possibly sterilizing immunity in some cases. Mendoza et al described 4 ECs who had weakly positive HIV western blots and proviral DNA levels that were 50-fold lower than that seen in traditional ECs [58]. Canouii et al found a subset of ECs who always had undetectable viral loads and had significantly lower proviral DNA levels and T cell activation than EC with occasional blips [59]. Zaunders et al recently described a member of the original Sydney Blood Bank Cohort who was infected with attenuated virus and currently has weak antibody responses and undetectable levels of proviral DNA in peripheral blood [60] and Casado and colleagues described 3 subjects who had been infected for more than 20 years who had extremely low levels of cell associated HIV DNA and RNA, and significantly lower levels of proviral diversity than traditional ECs [61]. Kwaa et al described a 200-fold variation in the level of intact proviral DNA that could be detected in the IPDA in ECs. One subject who has routinely had negative culture assays over greater than 10 years also had undetectable proviral DNA levels [26]. Jiang et al identified 2 EC who have extremely low levels of intact proviral DNA. A single intact proviral clone was detected from 1.02 billion CD4+ T cells in one subject and no detectable intact proviral clones were seen in 1.5 billion PBMCs from the other. Furthermore, they were unable to culture virus from more than 340 million CD4+ T cells and the IPDA did not detect intact DNA in a sample of 14 million CD4+ T cells [27]. These cases raise the question of whether a sterilizing cure has been achieved in some ECs. While transplantation with stem cells from donors with the delta 32 CCR5 mutation has resulted in possible sterilizing cures in 2 cases [62–63], we have learned that other interventions that dramatically reduce the number of latently infected CD4+ T cells in patients on ART do not lead to a cure [64–66]. A sterilizing cure is almost impossible to prove in elite controllers since the most stringent test of cure, a failure of viral rebound during a prolonged treatment interruption, cannot be applied to these subjects as the very definition of elite control is based on patients being aviremic without ART.

5. Implications for cure strategies

Overall it appears that most ECs have limited viral reservoirs that are controlled by the host immune response. The high percentage of CD4+ T cells with clonally expanded provirus and/or with provirus integrated in intergenic sites or sites with heterochromation features is probably a consequence of elite control rather than a cause. There have been very few studies of ECs during primary infection, but it appears that many of these patients never develop the high levels of viremia that is often seen in CPs and control viral replication shortly after infection [19,67–69]. The mechanism of control is unclear but may be due to rapid expansion of HIV-specific CD8+ T cells. Natural killer cells were also found to inhibit viral replication effectively during primary infection in one HIV controller [19], but more studies are needed to confirm that this is a common mechanism of early control. In one study where control of viral replication was regained following chemotherapy, the rate of viral decay was similar to that seen in subjects started on ART [20]. Treatment with ART in primary infection may be a way to mimic this rapid control and should dramatically limit the size of the viral reservoir [66,70,71] and in some cases lead to post-treatment control [72–74]. The induction of a potent T cell response or some other form of immunotherapy such as immunotoxin therapy [75] could lead to the selective elimination of cells harboring transcriptionally competent provirus. This response will probably be most effective very early in the course of infection when infected CD4+ T cells are still expressing HIV antigens and have not reached true latency (Fig. 2). Complete eradication of SIV appears to have been accomplished in macaques who were treated with a CMV vector-based vaccine that triggered very potent atypical SIV-specific CD8+ T cells prior to infection and this serves as an important proof of concept of the effect of early immune therapy on viral reservoirs [76]. A long-lasting immune response may be needed to eliminate any residual latently infected cell that reactivates after immune activation. This may require strategies such as repeated therapeutic vaccination or adoptive transfer of chimeric antigen receptor T cells [77]

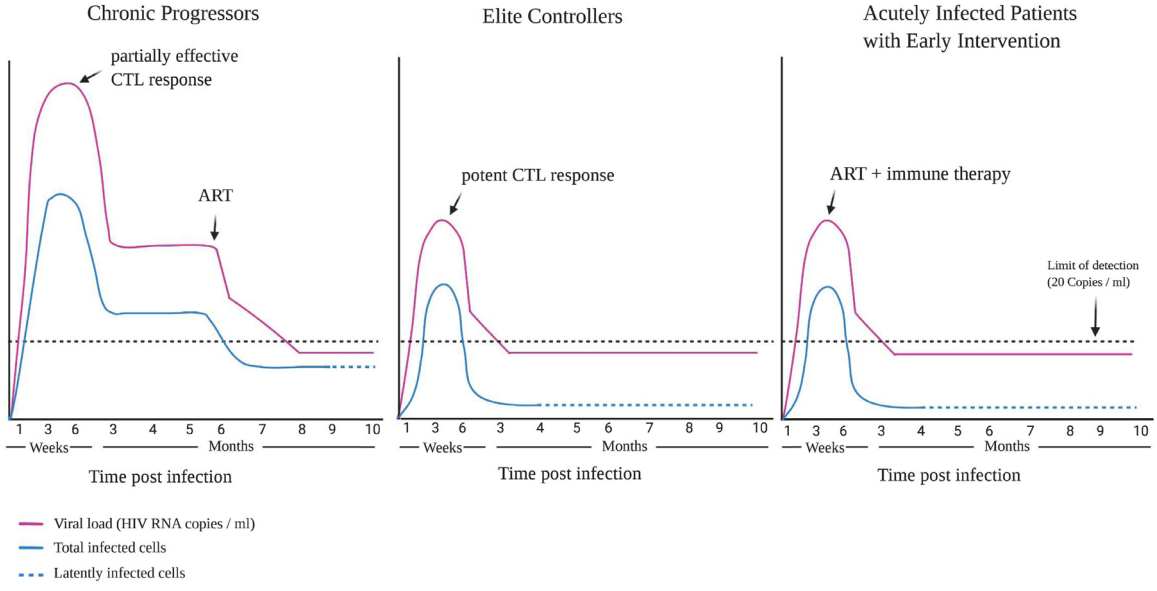

Fig. 2. HIV viremia and viral reservoirs in different groups of patients. Primary infection in chronic progressors is defined by very high levels of viremia leading to widespread seeding of viral reservoirs. A partially effective immune response leads to the lowering of the viral load to a set point. The introduction of ART inhibits viral replication to undetectable viral loads and reduces the number of infected CD4+ T cells. Elite controllers have much lower viral loads during primary infection and rapid control of viral replication leading to markedly lower number of infected cells. Treatment of patients with ART during primary infection also leads to lower levels of viremia and a reduction in the number of infected cells. Immunotherapy started at this time should lead to a further reduction in the size of the viral reservoir and potentially to a functional cure.

or infusions with broadly neutralizing antibodies [78]. Immune responses that are capable of penetrating sanctuaries such as B cell follicles will probably be needed to eliminate the source of ongoing viral replication seen in ECs. This will lead to enrichment of cells that harbor proviruses that are in a state of deep latency and are unlikely to produce infectious virus even when they become activated. It will probably be more challenging to block and lock proviruses that are already integrated at sites that favor transcription although a recent study that used the Tat inhibitor didehydro-Cortistatin A to mediate epigenetic silencing of the HIV promoter in infected humanized mice on ART suggests this approach may be promising [79]. Exceptional elite controllers who have very limited viral reservoirs in peripheral blood have been described, but before this is accepted as a sign of viral eradication, one must prove that there is no evidence of ongoing replication and viral evolution at other sites.

6. Outstanding questions

Most studies of viral reservoirs in elite controllers have focused on peripheral CD4+ T cells and it will be important to analyze myeloid cells, lymph nodes and other compartments. Very few studies have analyzed the reservoir during primary infection and it is not known whether the rate of decay of infected cells in this phase is similar to that seen after the initiation of ART in CPs. The mechanism of control of the viral reservoir in ECs who have weak HIV-specific CD8+ T cell responses is still unknown. This may inform strategies for CPs who do not have protective HLA alleles.

Search Strategy and Selection Criteria. Data for this Review were identified by searches of PubMed with search terms including combinations of HIV, controllers, elite, reservoirs. Search terms reservoir, cure and remission were also used to access the most recent articles on HIV cure strategies for CPs

Author contributions

BAW, AKK and JNB all searched the literature and wrote the paper. BAW made the Fig.s.

Fig.s/Tables: Both Fig.s are original and have not been published previously. **Figures were created with BioRender.Com**

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